

# Topological Distribution of Two Connexin32 Antigenic Sites in Intact and Split Rodent Hepatocyte Gap Junctions

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**Abstract.** The membrane topology of connexin32, a principal polypeptide of gap junctions in diverse cell types, has been studied in rat and mouse hepatocyte gap junctions using site-specific antisera raised against synthetic oligopeptides corresponding to amino acid sequences deduced from cDNA clones. Based on published hydropathicity maps and identified protease-sensitive cleavage sites, oligopeptides were synthesized corresponding to two hydrophilic domains of connexin32, one predicted to face the cytoplasm, the other predicted to be directed extracellularly. Antisera were raised to keyhole limpet hemocyanin conjugates of the oligopeptides and used to map the distribution of their antigens using indirect immunocytochemistry on isolated gap junctions. The results directly demonstrated the cytoplasmic orientation of an antigen contained within amino acids 98–124 of the connexin32 sequence. The extracellular space in intact, isolated gap junctions is too small to permit binding of anti-

body molecules, necessitating the experimental separation of the junctional membranes to expose their extracellular surfaces using a urea/alkali procedure. While an antigen contained within amino acids 164–189 was visualized on the extracellular surfaces of some of the separated junctional membranes, variability in the observations and in the splitting procedure left ambiguities concerning the biological relevance of the observations after the denaturing conditions necessary to separate the junctional membranes. Using a different approach, however, the antigen could be exposed in intact liver using a hypertonic disaccharide junction-splitting procedure. The period of time of antigen exposure at the cell surface appears to peak at 30 s and disappear by 2–4 min. Taken together, these data demonstrate the extracellular orientation of an antigen contained within amino acids 164–189, which may be involved in cell–cell interaction within the gap junction.

COMPLIMENTARY DNAs coding for gap junction proteins have been cloned from liver, heart, and lens libraries (Paul, 1986; Kumar and Gilula, 1986; Beyer et al., 1987, 1988). The protein coded by the liver sequence has been localized to gap junctions not only joining hepatocytes, but also joining other cell types in other organs (Hertzberg and Skibbens, 1984; Dermietzel et al., 1984; Paul, 1985). The predicted molecular mass of the protein from rat liver is 32,007, and it has been suggested that the protein be called connexin32 to distinguish this junctional protein from homologous proteins whose cDNAs have been cloned from myocardial and lens libraries (Beyer et al., 1987, 1988).

Zimmer et al. (1987) have studied the topology of rat connexin32 by examining protease products generated by digestion of morphologically intact isolated rat liver gap junctions and junctions centrosymmetrically split into single membranes using a urea-alkaline procedure (Manjunath et al., 1984). These authors presented clear evidence for the cytoplasmic location of the carboxy terminus of the connexin32 molecule and were able to demonstrate a unique lys-X endoprotease cleavage site dividing the molecule into 10 and 17 kD fragments. TPCK-trypsin also cleaves at this unique site, demonstrating that this region of the molecule

faces the protease-accessible cytoplasmic surface. Zimmer et al. (1987) presented a model of the topology of connexin32 with respect to the junctional lipid bilayer based on their findings. In addition to the data demonstrating protease-accessible sites reviewed above, this model is consistent with hydropathicity plots generated from the amino acid sequence data available from the cDNA clones (Paul, 1986). The model is redrawn in Fig. 1 and specifies both the amino and carboxyl terminals of connexin32 facing the cytoplasm. There are four transmembrane hydrophobic domains, two extracellular domains, and one central cytoplasmic domain. Zimmer et al. (1987) have provided direct evidence for the location of both the COOH terminus and lysine-124 at the cytoplasmic junctional surfaces. However, there are as yet no published data directly demonstrating that there are two transmembrane regions and an extracellular domain between these two separate protease-cleavage sites. Nor are there direct data demonstrating either the presence of a second extracellular domain (drawn here as residues 43–72) or the cytoplasmic disposition of the NH<sub>2</sub> terminus.

We have produced polyclonal antisera using as antigen synthetic oligopeptides based on predicted amino acid sequences for two domains within the connexin32 molecule.

The location of these domains are indicated on the model in Fig. 1. One is localized in the predicted central cytoplasmic domain and the other in a predicted extracellular domain. The central cytoplasmic domain antigen is a 27-mer that corresponds to residues 98–124 of the rat connexin32 sequence. The extracellular domain antigen is a 26-mer that corresponds to residues 164–189. To provide additional evidence for the model drawn by Zimmer et al. (1987), the antisera have been used to localize the sidedness of their epitopes with respect to the junctional membrane by indirect immunocytochemistry. Since possible rearrangements of the protein as a result of the experimental protocol necessary to split the gap junctions cannot be determined, the gap junctions were split by two completely different protocols: immunocytochemistry was performed on intact and urea/alkaline-split isolated rat hepatocyte gap junctions, and immunohistochemistry was performed on gap junctions joining hepatocytes in whole liver using the hypertonic disaccharide junction-splitting technique (Goodenough and Gilula, 1974). The data confirm the cytoplasmic location of the 98–124 region of the protein and provide evidence both for the extracellular location of an epitope contained in residues 164–189 and, thus, for at least two intervening membrane-spanning regions.

## Materials and Methods

### Reagents

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

The following peptides were synthesized by Bachem, Inc. (Torrance, CA). Peptide 98/124: Gln-Gln-His-Ile-Glu-Lys-Lys-Met-Leu-Arg-Leu-Glu-Gly-His-Gly-Asp-Pro-Leu-His-Leu-Glu-Glu-Val-Lys-Arg-His-Lys. Peptide 98/124 was 3287 D and was demonstrated by the supplier to be >97% pure by HPLC and amino acid analysis. Chromatography was performed on a Vydac C18 column after reduction with dithiothreitol (DTT). The sample was eluted with a gradient of 5–100% acetonitrile plus 0.1% trifluoroacetic acid over 20 min. Peptide 164/189: Arg-Leu-Val-Lys-Cys-Glu-Ala-Phe-Pro-Cys-Pro-Asn-Thr-Val-Asp-Cys-Phe-Val-Ser-Arg-Pro-Thr-Glu-Lys-Thr-Val. Peptide 164/189 was 2823 D and >98% pure by the same criteria above. Keyhole limpet hemocyanin (KLH)<sup>1</sup> was purchased from Pacific Bio Marine Laboratories, Inc. (Venice, CA).

### Preparation of Antisera

The peptides were coupled to the KLH by the following procedure, attempting to achieve a 30:1 molar ratio of peptide to KLH. 780 nmols of peptide were reconstituted in 400  $\mu$ l PBS. 26 nmols of KLH in 250  $\mu$ l of PBS were added. 5  $\mu$ l of 25% glutaraldehyde (Sigma Chemical Co., St. Louis, MO) were added and the resultant solution incubated overnight at room temperature. Some turbidity was observed to develop. No efforts were made to further purify the conjugates.

For primary immunizations, peptide-KLH conjugate containing 200  $\mu$ g peptide was emulsified in Freund's Complete Adjuvant (Gibco Laboratories, Grand Island, NY) and was injected in duplicate into preimmune bled, female New Zealand white rabbits. All injections were made in multiple paravertebral intradermal sites. After 30 d, the rabbits were boosted with KLH-peptide in incomplete Freund's and were bled 7–10 d later. Rabbits were boosted 5 times thereafter at monthly intervals. Antisera were screened against peptide by absorbing unconjugated peptide to nitrocellulose, blocking with 5% dehydrated milk reconstituted in PBS (BLOTTO), and reacting with the test antiserum followed by visualization with peroxidase-conjugated goat anti-rabbit secondary according to standard techniques. Antisera were tested for reactivity to connexin32 by Western analysis of isolated rat liver gap junctions prepared according to Baker et al. (1983). Isolated junctions were subjected to SDS-PAGE according to Laemmli (1970) in 12%

gels and electroblotted to nitrocellulose in gel running buffer containing 20% methanol but without SDS. Electroblothing was performed for 1 h at 75 V with 10 cm electrode spacing at 4°C. After transfer, nitrocellulose filters were blocked in 10% BLOTTO in PBS for 1 h at 22°C. Blocked filters were washed briefly in PBS then incubated overnight at 4°C in primary antiserum diluted in PBS containing 0.5% Tween-20 (PBS-Tw). Monoclonal M12.13 culture supernatant was diluted 1:10, anti-98/124 was diluted 1:10,000, and anti-164/189 was diluted 1:1,000. Filters were washed 3 times for 5 min with shaking at 22°C and incubated with <sup>125</sup>I-labeled protein A (50  $\mu$ Ci/ $\mu$ g,  $5 \times 10^5$  cpm/ml) in PBS-Tw for 1 h at 4°C. Filters were washed as described above and exposed to XAR-5 film with intensifying screen at –80°C overnight.

### Monoclonal Antibodies

An anticonnexin32 monoclonal antibody (R5.21C) was used to determine the sidedness of the junctional membranes after urea/alkali splitting. This antibody has been characterized previously and has been shown to bind to the cytoplasmic surfaces of isolated gap junctions (Stevenson et al., 1986). A new anticonnexin32 monoclonal was used in the sucrose-splitting experiments. The monoclonal was raised by immunizing BALB/c mice (Charles River Breeding Laboratory, Wilmington, MA) intraperitoneally with isolated rat junctional complexes, prepared as described by Stevenson and Goodenough (1984). The myeloma line, fusion, and screening protocols were identical to those described (Stevenson et al., 1986). The resultant mouse hybridoma was designated M12.13. As shown by Western analysis in Results, this monoclonal recognizes the "27-kD" major protein in hepatocyte gap junctions, characteristic of connexin32. Indirect immunocytochemistry revealed that M12.13 recognized its antigen on the cytoplasmic surface of isolated rat or mouse gap junctions, indistinguishable from R5.21C staining (data not shown). We have not determined which epitopes these monoclonal antibodies are directed against. However, neither reacts with either of the oligopeptides described here.

### Sucrose Perfusion of Whole Mouse Liver

Sucrose splitting of mouse hepatocyte gap junctions was performed as follows. 0.1 ml of 300 U/ml heparin (Sigma Chemical Co.) in 155 mM NaCl was injected with an 18-gauge needle into the inferior vena cava of mice anesthetized with sodium pentobarbital (Abbott Labs, North Chicago, IL) and the needle left in place. The portal vein was then cannulated with a 22-gauge needle and the liver perfused at 40 cm H<sub>2</sub>O pressure with 10 ml of 155 mM NaCl with 3 mM EGTA, pH 7.4. The 18-gauge needle in the inferior vena cava was simultaneously removed to allow fluid outflow. The livers were then perfused with 0.5 M sucrose in 155 mM NaCl and 10 mM CaCl<sub>2</sub>, pH 7.4. The addition of EGTA and CaCl<sub>2</sub> follows the demonstration by Peracchia (1977) that gap junction splitting is more uniform after inclusion of these reagents. Sucrose perfusion times were 15 s to 4 min, at which time the tip of the right hepatic lobe was snipped off with scissors and frozen as rapidly as possible in liquid freon cooled with liquid nitrogen. The cutting and freezing step added a variable 10–40 s to the sucrose-exposure time. 6  $\mu$  frozen sections of the perfused, unfixed livers were immersed in absolute acetone at –20°C for 3 min, before antibody incubation. Immunohistochemistry was performed by overnight incubation at 4°C of the sections with 1:500 and 1:1,000 dilutions of unfractionated immune and preimmune sera, or straight hybridoma culture supernatant. All secondary antisera were used at 1:500 dilution (Boehringer Mannheim Biochemicals, Indianapolis, IN).

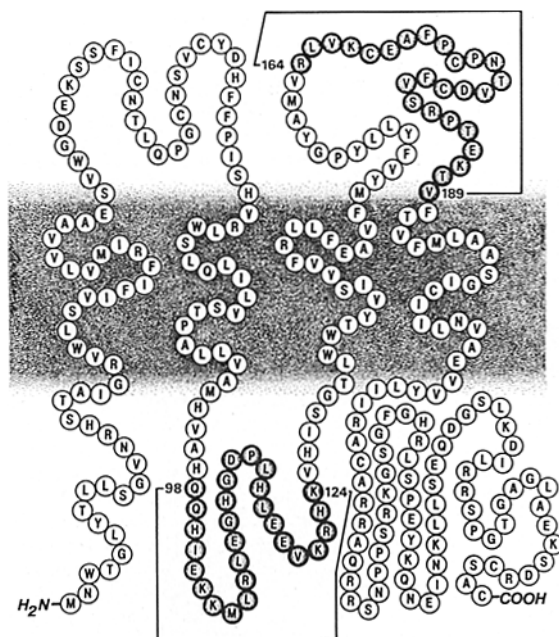
Immunocytochemistry was performed on isolated rat liver gap junctions (Baker et al., 1983) using the methods of Paul and Goodenough (1983). Gold-labeled secondary antisera were obtained from Janssen Life Sciences Products (Piscataway, NJ) and used at full strength after exhaustive absorption with isolated rat liver gap junctions. Isolated liver gap junctions were split according to the methods of Manjunath et al. (1984), using both 15 min and 1 h exposures to the urea/alkali splitting solutions. Negative stain electron microscopy of the split junctions was performed as described (Fallon and Goodenough, 1981).

## Results

### Western Analysis

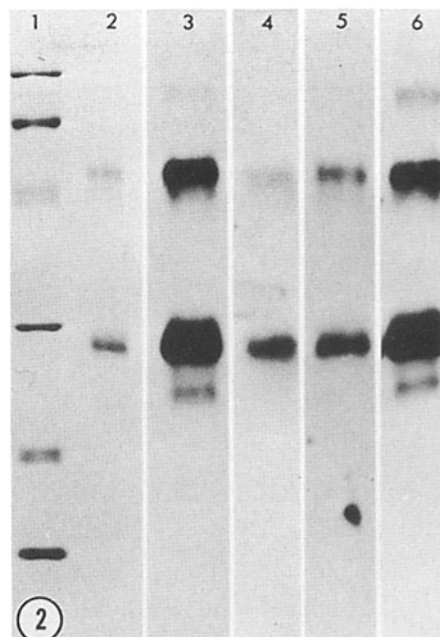
Western blot analysis of the antipeptide antisera and the monoclonal M12.13 is shown in Fig. 2. Lanes 1 and 2 show molecular mass standards and  $\sim$ 1  $\mu$ g of an isolated rat liver

1. Abbreviation used in this paper: KLH, keyhole limpet hemocyanin.



**Figure 1.** The amino acid sequence of connexin32, in single letter code, has been folded through a single junctional membrane as suggested by published proteolysis studies and hydropathicity plots. The hydrophobic core of the junctional membrane is represented by the stippled band across the figure, with extracellular (gap) at the top, and intracellular (cytoplasmic), containing both the NH<sub>2</sub> and COOH termini, at the bottom. The membrane stippling is graded at the two surfaces to indicate ambiguity as to the precise location where the polypeptide enters or leaves the membrane matrix. Smaller folds within the polypeptide chain serve only to fit the drawing in the space; no secondary structure is implied. The segments of the polypeptide that were synthesized as oligopeptides for antisera production are shaded, corresponding to amino acids 98–124 and 164–189.

gap junction preparation, respectively, stained with Coomassie Brilliant Blue. Lanes 3–6 are immunoblots containing ~10 ng of isolated rat liver gap junctions reacted with differ-

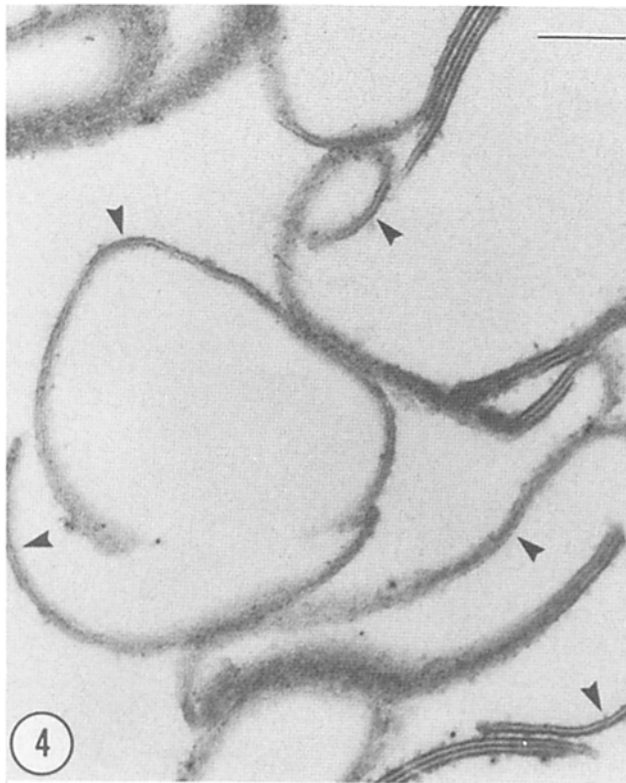


**Figure 2.** Immunoblot analysis of the antisera. (Lanes 1 and 2) Coomassie Blue-stained gel used for transfer to nitrocellulose. Lane 1, molecular mass standards; lane 2, isolated rat hepatocyte gap junctions. (Lanes 3–6) Immune replicas of isolated rat gap junction preparations. Lane 3, anti-98/124; lanes 4 and 5, two different anti-164/189 antisera; lane 6, monoclonal antibody M12.13.

ent immune reagents. Lane 3 was reacted with anti-98/124, lanes 4 and 5 with two different anti-164/189s, and lane 6 with the monoclonal antibody, M12.13. All reagents reacted with the 27-kD principal polypeptide of the rat liver gap junctions, in addition to the aggregate at ~47 kD. The reagents directed against “cytoplasmic” epitopes, in lanes 3 and 6, react with presumed proteolysis fragments not observed with the two reagents directed against “extracellular” epitopes (lanes 4 and 5). Published immunoblots with other poly-



**Figure 3.** Isolated rat hepatocyte gap junctions were treated with the urea/alkali procedure then negatively stained with uranyl acetate. *a* contains an image of an unsplit gap junction, identified by its double stain-excluding edge at a fold (arrow) and the lattice of connexons. *b* shows two split junctions, which show only single stain-excluding edges at folds (arrowheads). The right-hand split junction contains a lattice of connexons while the left-hand junctional membrane shows extreme lattice disorder, such that the connexons are no longer clearly visible. The white arrows indicate aggregated material also present in the junctional preparations after urea/alkali treatment. Bar, 100 nm.

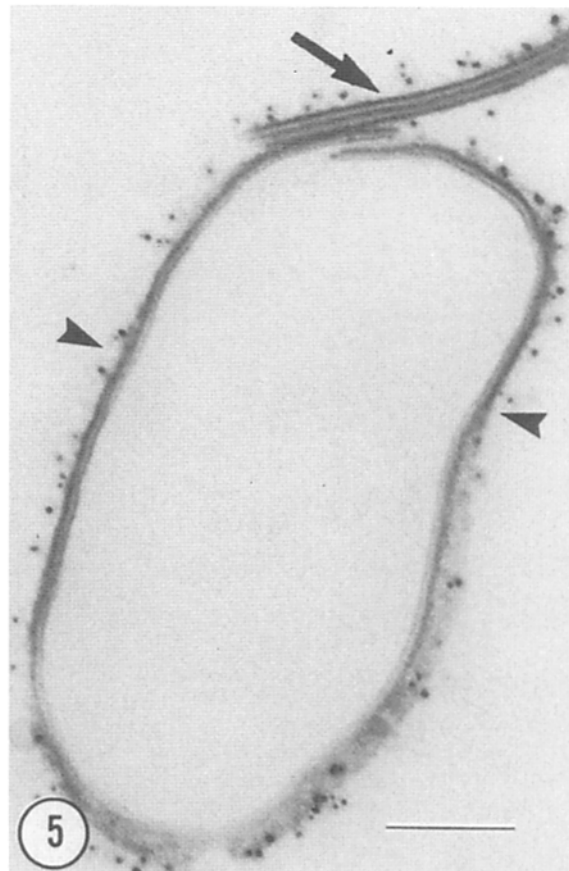


**Figure 4.** Electron micrograph of urea/alkali-split gap junctions reacted with preimmune anti-98/124. The variability of gap junctional splitting seen in the negatively stained images in Fig. 3 is also evident in this image of thin sections. The figure shows intact junctions and curved single membranes (*arrowheads*), none of which interact with the preimmune serum. Similar background staining was demonstrable with the other preimmune sera and nonimmune culture supernatant (data not shown). Bar, 100 nm.

clonal antisera which recognize the cytoplasmic surfaces of isolated gap junction, also recognize this and other proteolytic fragments (Paul, 1986).

#### **Structure of Urea/Alkali-split Isolated Gap Junctions**

Isolated rat liver gap junctions were used for immunocytochemistry both intact and after splitting with the urea/alkali procedure. After the splitting reaction, the gap junction preparations were found to be heterogeneous by both negative staining and thin-section criteria. Since the denaturing conditions of the urea/alkali procedure may have destroyed the protein topology of the connexin32 molecule, negative staining was employed to visualize the integrity of the lattice of connexons in the split junctional membranes. In negative stain (Fig. 3 *a*), unsplit gap junctions showed the double stain-excluding profile at their folded edges (*arrow*), characteristic negatively stained, isolated gap junctions (Goodenough, 1976). The split junctions in Fig. 3 *b* showed only a single stain-excluding edge where the half-junctions folded (*arrowheads*). One of the split junctions in Fig. 3 *b* showed a lattice of connexons, while the other showed a tendency to begin fragmentation and individual connexons were poorly visible. Aggregates of material were also evident (*white arrows*). Taken together, these images indicated that the splitting of rat liver junctions by the urea-alkali method was not

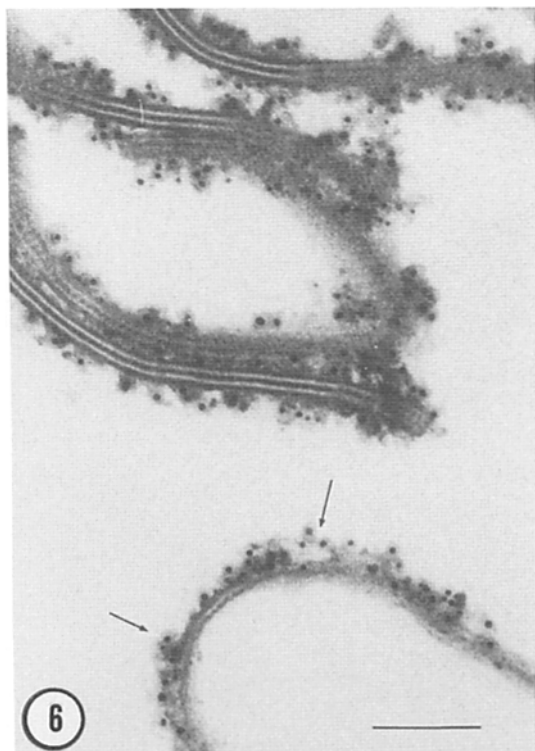


**Figure 5.** Electron micrograph of urea/alkali-split gap junctions reacted with monoclonal antibody R5.21C. The variability of gap junctional splitting seen in the negatively stained images in Fig. 3 is also evident in these images of thin section. The monoclonal antibody staining is seen on the cytoplasmic surfaces of the unsplit gap junction (*arrow*) and on the convex surfaces of the split junctional membranes, marking this surface as cytoplasmic. Bar, 100 nm.

uniform, a relevant point in the interpretation of the immunocytochemistry data presented below. Some of the junctions remained unsplit, some split and retained a lattice of connexons, while others split and disordered their lattice of connexons. Aggregates of material appeared in variable amounts. A similar spectrum of images was produced whether the urea/alkali was performed for 15 min or 1 h.

#### **Immunocytochemistry**

Fig. 4 shows staining of the split junction preparation with anti-164/189 preimmune serum, as visualized by thin-section electron microscopy showing both intact junctions and single membranes. Fig. 5 shows immunocytochemistry using monoclonal antibody R5.21C. Note in both figures (*arrowheads*) that when the gap junctions split into single membranes, the membranes curled, producing concave and convex surfaces. As shown previously (Stevenson et al., 1986), R5.21C stained the cytoplasmic surfaces of the intact, unsplit gap junctions (*arrow*, Fig. 5). The monoclonal stained only the convex face of the single membranes, demonstrating that the half-junctions curled with their cytoplasmic surfaces convex. This staining was absolute: of 33 single membranes



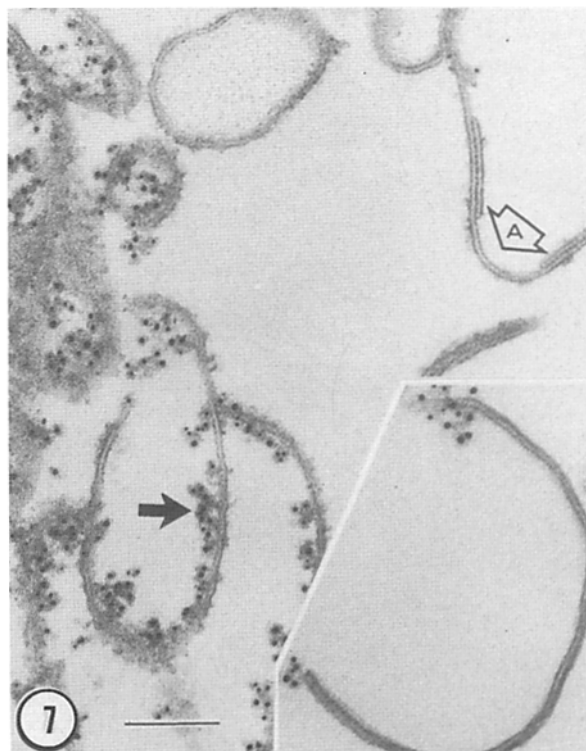
**Figure 6.** An electron micrograph of the urea/alkali-split gap junctions stained with anti-98/124. Intense staining is evident on the cytoplasmic surfaces of the unsplit junctions and on the convex (cytoplasmic) surface of a split junctional membrane. No staining is observed on the exposed, concave surfaces of split junctional membranes. Bar, 100 nm.

cut in unambiguous cross section from 20 electron micrographs, all 33 were labeled on their convex surfaces. No exceptions were ever observed. This consistent curvature and antibody staining rigorously established the convex surface of single membranes as the cytoplasmic surface. This consistent curvature of the split junctional membranes was also documented by Zimmer et al. (1987).

Fig. 6 is an electron micrograph of a urea/alkali-split isolated gap junction preparation stained with anti-98/124 immune serum. Colloidal gold particles festooned the cytoplasmic surfaces of the unsplit gap junctions, demonstrating that this epitope was cytoplasmic, as drawn in Fig. 1. Fig. 6 reveals that the gold was also localized to the convex (cytoplasmic) surfaces of the single, split junctional profiles (arrows). As with R5.21C, all nonobliquely sectioned single membrane profiles stained with the anti-98/124 antiserum on their convex surfaces; no single membranes ever stained on their concave surfaces with this reagent.

The staining of the urea/alkali-split gap junction preparations with the anti-164/189 immune serum had both consistent and variable features, as documented in Figs. 7 and 8. The consistent features were that positive staining was never observed on the cytoplasmic junctional surfaces, either on the intact or split profiles. In addition, collections of vesicles and aggregated material were always observed to stain (Fig. 8, *inset*).

In comparison to these consistent results, anti-164/189



**Figure 7.** Electron micrograph of the urea/alkali-split gap junctions stained with anti-164/189. The cytoplasmic surfaces of unsplit gap junctions and the convex (cytoplasmic) surfaces of the split junctional membranes were never observed to stain. The concave (extracellular) surfaces of the split junctional membranes were observed to stain in some cases (*solid arrow*) and not stain in others (*open arrow, A*). The free edges of the split junctional membranes were often observed to stain (*inset*). Bar, 100 nm.

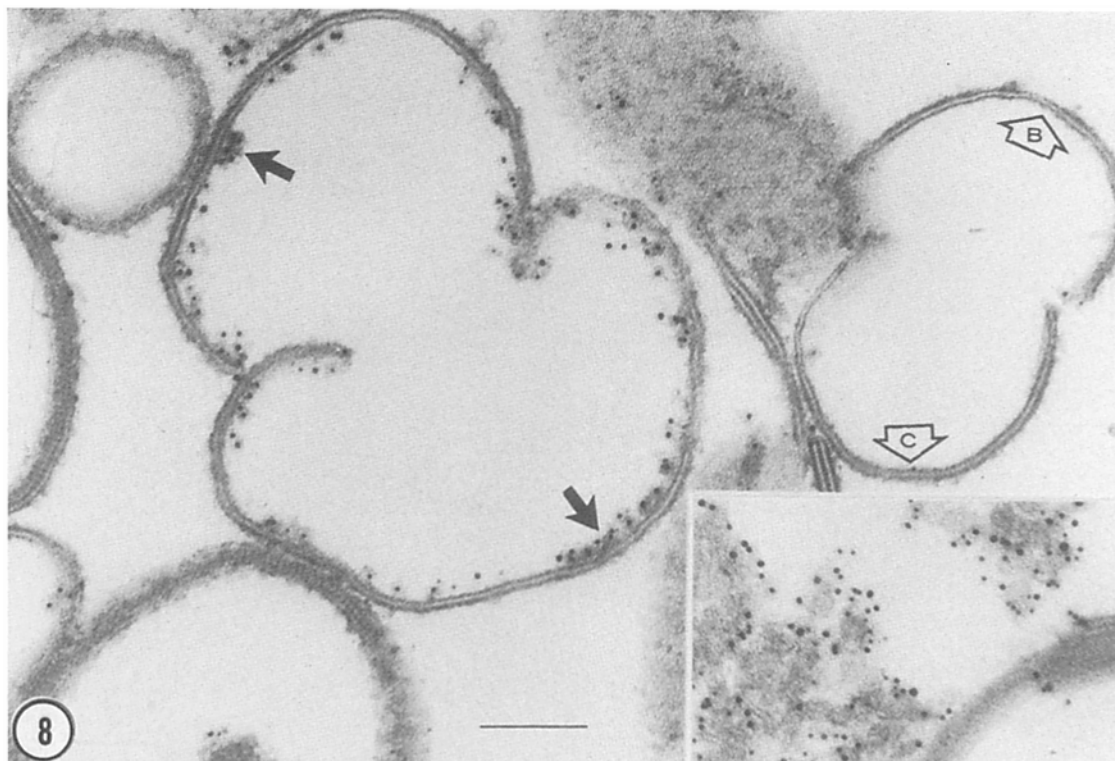
also showed variable staining of the urea/alkali-split gap junction preparations. Gold labeling was observed on ~20% of the concave, extracellular surfaces of the split junctions (*closed arrows*, Figs. 7 and 8), while ~80% of the single membranes either did not stain at all (*open arrows A, B, and C*, Figs. 7 and 8), or stained only at their free edges (*inset*, Fig. 7). This variability was observed not only from specimen to specimen, but also within different areas of a single specimen. The centrifuge pellets were always oriented before sectioning; this did not provide an explanation for the variability.

### Immunohistochemistry and Sucrose Splitting

Immunohistochemistry of frozen sections of unfixed mouse liver stained with anti-98/124 antiserum (data not shown) revealed macular staining between adjacent hepatocytes characteristic of gap junction staining published in several laboratories (Hertzberg and Skibbens, 1984; Dermietzel et al., 1984; Paul, 1985, 1986). Anti-164/189 antiserum did not produce any specific staining on cryosections at any dilution, indicating that the epitope was not exposed in these specimens (data not shown).

Perfusion of the livers with hypertonic sucrose followed by immunohistochemistry with anti-164/189 resulted in macular staining associated with the hepatocyte cell surfaces (Fig.





**Figure 8.** Electron micrograph of the urea/alkali-split gap junctions stained with anti-164/189. The cytoplasmic surfaces of unsplit gap junctions and the convex (cytoplasmic) surfaces of the split junctional membranes were never observed to stain. The concave (extracellular) surfaces of the split junctional membranes were observed to stain in some cases (*solid arrows*) and not stain in others (*open arrows, B and C*). Stained and unstained split junctions were observed side by side in the same specimens. The clumps of vesicles were always observed to stain (*inset*). Bar, 100 nm.

9 *a*). To demonstrate that the anti-164/189 staining exposed by the sucrose perfusion corresponded to gap junctions and not to sticky regions generated by the hypertonic cell separation, frozen sections of sucrose-perfused mouse liver were double-stained with anti-164/189 (Fig. 9 *a*) and with the M12.13 monoclonal antibody (Fig. 9 *b*), which recognized the cytoplasmic side of the gap junction. There was a one-to-one correspondence between the junctional staining seen with these two reagents, indicating that the sucrose-induced antigenic sites were contained in the same junctional plaques seen with an intracellularly directed antibody.

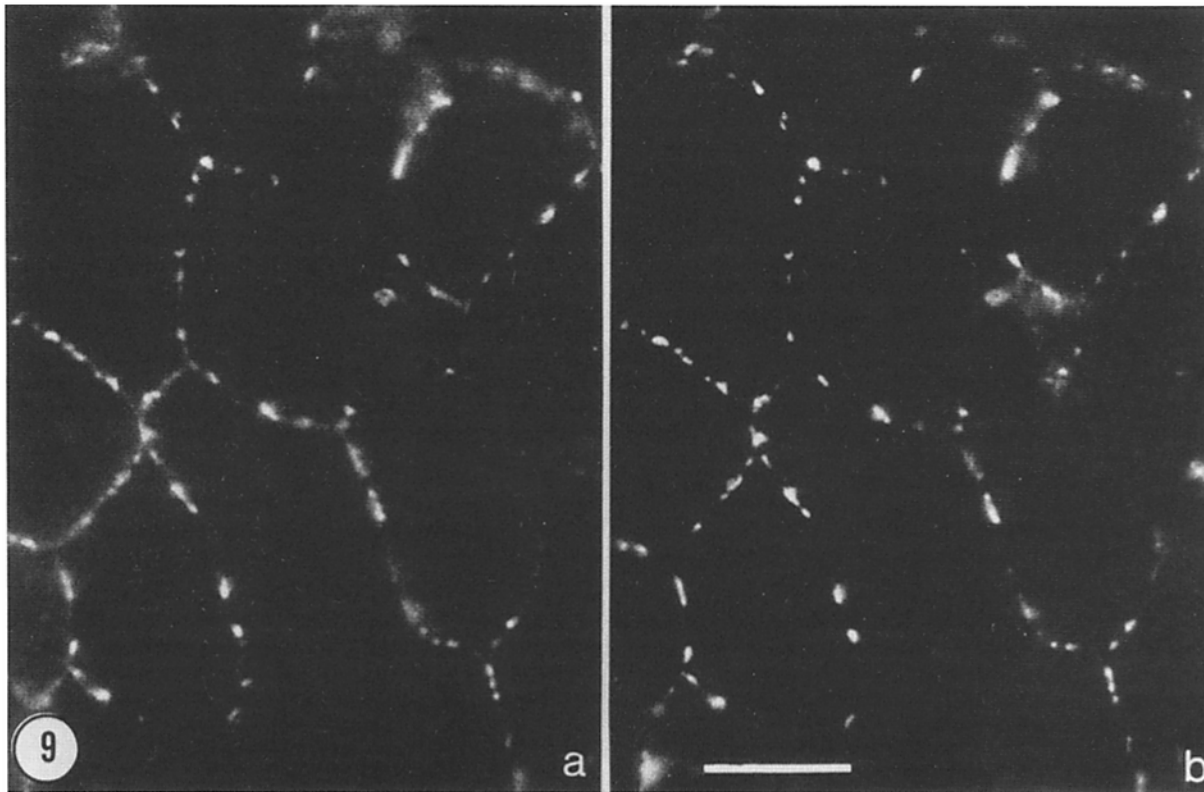
The period of time during which the 164/189 epitope was exposed appeared to be very short. 14 livers were perfused for different times. 3 out of 4 at the 30-s time point showed detectable anti-164/189 staining, 1 out of 4 showed staining at 45 s, 0 of 4 were positive at 1 min, and 0 of 2 were positive at 2 min. Due to ambiguities in the handling times of each liver and variabilities in the degree and efficiency of perfusion, precise timing of the junctional splitting was difficult to quantitate. Nonetheless, it appeared that the 164/189 epitope was exposed only briefly after sucrose perfusion, with a peak in exposure  $\sim 30$  s after plasmolysis.

## Discussion

Antisera have been raised using synthetic oligopeptides that corresponded to defined regions of the predicted amino acid sequence of rat connexin32, the principal polypeptide of rat

liver gap junctions. The oligopeptides were selected on the basis of hydropathicity plots of predicted amino acid sequence data from a cDNA clone and a model proposed by Zimmer et al. (1987), which was redrawn in Fig. 1. Oligopeptide 98/124 was predicted to face the cytoplasmic surface of the gap junction, and light and electron microscopic data presented here using an anti-98/124 antiserum directly demonstrated the cytoplasmic localization of this portion of the connexin32 molecule.

The localization of oligopeptide 164/189 was less clear. Due to the narrow extracellular space between the apposed junctional membranes, antibody molecules are sterically hindered from binding to their antigens (Goodenough and Revel, 1971), except perhaps at the edges of isolated junctional plaques. Attempts to expose epitopes recognized by anti-164/189 antiserum by splitting the isolated junctions with a urea/alkali procedure produced variable results. Negative staining demonstrated that the splitting reaction was nonuniform with different junctions showing different responses to identical conditions. In some split junctions the lattice of connexons appeared intact, while in others the lattice appeared disordered or destroyed. The staining with the anti-164/189 antiserum was also variable. Some of the split junctional membranes stained with anti-164/189 on their extracellular surfaces in support of the model in Fig. 1, but many of the single membranes did not. Staining was variably seen on the edges of isolated junctions, and on the edges of membranes from split junctions.



**Figure 9.** Fluorescence photograph of an unfixed frozen section of mouse liver perfused with 155 mM NaCl + 3 mM EDTA, subsequently perfused with 0.5 M sucrose + 10 mM CaCl<sub>2</sub> for 30 s. The section was double-stained with anti-164/189 (a) and with monoclonal antibody M12.13 (b). The anti-164/189 staining (visualized with rhodamine goat anti-rabbit) is precisely matched by the M12.13 staining (visualized with FITC-labeled goat anti-mouse). Apparent differences in relative intensity of individual macula, visualized with the different antisera, are partially due to differences in focal plane between the pair of exposures. Bar, 10  $\mu$ m.

It was not possible to conclude from these data that the 164/189 epitope faced the extracellular space in the intact gap junction. The urea/alkali procedure inconsistently and variably split the liver junctions. The resulting heterogeneous collection of unsplit, split, and fragmented junctional membranes did not show uniform staining with the anti-164/189 reagent, and for this reason the urea/alkali procedure may not be useful for some topological studies. The disruptive conditions necessary to split the junctions may denature the tertiary or quaternary structure of either connexin32 or the connexon and cause portions of connexin32 molecule, normally sequestered within the junctional membranes, to become artifactually exposed at the extracellular surfaces. Conversely, the observed staining of some of the split membranes may reflect the true extracellular disposition of the 164/189 domain of the connexin32 molecule, and the absence of staining reflect changes induced after splitting, rendering the epitope unavailable for antibody binding.

These data are consistent with the observations of Zimmer et al. (1987). These authors also report variable splitting of the isolated junctions. The model in Fig. 1 shows potential proteolytic cleavage sites within the 164/189 loop that Zimmer et al. (1987) were unable to cleave in their urea/alkali-split gap junction specimens using exogenous proteases. This failure to cleave these sites may be related to the variability of splitting with the urea/alkali procedure.

To provide some additional experimental evidence in support of the extracellular disposition of the 164/189 domain

of the connexin32 molecule, intact mouse livers were perfused with hypertonic sucrose to split the gap junctions by means of a different mechanism than the urea/alkali procedure. At the light microscope level, the sucrose treatment was capable of exposing the 164/189 antigen at sites that exactly corresponded to the junctional plaques visualized by a cytoplasmically localized monoclonal antibody, M12.13. The sucrose-splitting protocol was difficult to control due to uneven perfusion of different hepatic lobules, different degrees of plasmolysis induced in the hepatocytes by the hypertonic conditions, and experimental timing of the freezing of the specimens within the very short time frame during which the effect was observed. Nonetheless, it was clear that it was possible to systematically expose the 164/189 antigen on all detectable junctional plaques if the timing centered on  $\sim 30$  s exposure to the hypertonic conditions. Since this localization has been carried out thus far only at the light microscope level, it is not possible to formally rule out the possibility that the 164/189 antigen has been exposed on the cytoplasmic surface of the sucrose-split gap junctions.

In summary, the topology of the connexin32 protein, which comprises a principal component of gap junctions found between hepatocytes and other cell types, has been investigated in this study. The location of an antigen contained between amino acids 98–124 was confirmed as facing the cytoplasm. A combination of cytochemical localization on urea/alkali-split, isolated gap junctions with immunohistochemical localization on sucrose-split gap junctions in situ

provided evidence that an antigen contained between amino acids 164–189 faced the extracellular space, and hence represented a portion of the connexin32 molecule that could participate directly in cell–cell interaction. If so, this portion of the molecule rapidly became inaccessible to extracellularly applied antisera after separation of the cells. This cysteine-rich region is highly conserved between different connexin molecules isolated from myocardium and lens (Beyer et al., 1987, 1988), and so may permit heteromolecular interaction between heterologous cell types expressing different connexin genes. Other features of the model, the cytoplasmic disposition of the NH<sub>2</sub> terminal and the extracellular location of the domain contained between residues 43 and 72, remain to be demonstrated in future studies.

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### References

- Baker, T. S., D. L. D. Caspar, C. J. Hollingshead, and D. A. Goodenough. 1983. Gap junction structures IV. Asymmetric features revealed by low-irradiation microscopy. *J. Cell Biol.* 96:204–216.
- Beyer, E. C., D. A. Goodenough, and D. L. Paul. 1988. The connexins: a family of related gap junction proteins. In *Gap Junctions*. E. L. Hertzberg and R. Johnson, editors. Alan R. Liss, Inc., New York. In press.
- Beyer, E. C., D. L. Paul, and D. A. Goodenough. 1987. Connexin43: a protein from rat heart homologous to a gap junction protein from liver. *J. Cell Biol.* 105:2621–2629.
- Dermietzel, R., A. Leibstein, U. Frixen, U. Janssen-Timmen, O. Traub, and K. Willecke. 1984. Gap junctions in several tissues share antigenic determinants with liver gap junctions. *EMBO (Eur. Mol. Biol. Organ.) J.* 3: 2261–2270.
- Fallon, R. F., and D. A. Goodenough. 1981. Five-hour half-life of mouse liver gap-junction protein. *J. Cell Biol.* 90:521–526.
- Goodenough, D. A. 1976. In vitro formation of gap junction vesicles. *J. Cell Biol.* 68:220–231.
- Goodenough, D. A., and N. B. Gilula. 1974. The splitting of hepatocyte gap junctions and zonulae occludentes with hypertonic disaccharides. *J. Cell Biol.* 61:575–590.
- Goodenough, D. A., and J. P. Revel. 1971. The permeability of isolated and in situ mouse hepatic gap junctions studied with enzymatic tracers. *J. Cell Biol.* 50:81–91.
- Hertzberg, E. L., and R. V. Skibbens. 1984. A protein homologous to the 27,000 dalton liver gap junction protein is present in a wide variety of species and tissues. *Cell.* 39:61–69.
- Kumar, N. M., and N. B. Gilula. 1986. Cloning and characterization of human and rat liver cDNAs coding for a gap junction protein. *J. Cell Biol.* 103: 767–776.
- Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680–688.
- Manjunath, C. K., G. E. Goings, and E. Page. 1984. Detergent sensitivity and splitting of isolated liver gap junctions. *J. Membr. Biol.* 78:147–155.
- Paul, D. L. 1985. Antibody against liver gap junction 27-kD protein is tissue specific and cross-reacts with a 54-kD protein. In *Gap Junctions*. M. V. L. Bennett and D. C. Spray, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Paul, D. L. 1986. Molecular cloning of cDNA for rat liver gap junction protein. *J. Cell Biol.* 103:123–134.
- Paul, D. L., and D. A. Goodenough. 1983. Preparation, characterization, and localization of antisera against bovine MP26, an integral protein from lens fiber plasma membrane. *J. Cell Biol.* 96:625–632.
- Peracchia, C. 1977. Gap junctions. Structural changes after uncoupling procedures. *J. Cell Biol.* 72:628–641.
- Stevenson, B. R., and D. A. Goodenough. 1984. Zonulae occludentes in junctional complex-enriched fractions from mouse liver: preliminary morphological and biochemical characterization. *J. Cell Biol.* 98:1209–1221.
- Stevenson, B. R., J. D. Siliciano, M. S. Mooseker, and D. A. Goodenough. 1986. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J. Cell Biol.* 103:755–766.
- Zimmer, D. B., C. R. Green, W. H. Evans, and N. B. Gilula. 1987. Topological analysis of the major protein in isolated intact rat liver gap junctions and gap junction-derived single membrane structures. *J. Biol. Chem.* 262:7751–7763.